Effect of *Carya illinoinensis, Quercus rubra* and *Smilax glyciphylla* extracts, pectin, and papain on the dental biofilm microorganisms

[Efecto de extractos de *Carya illinoinensis, Quercus rubra* y *Smilax glyciphylla*, pectina y papaina sobre los microorganismos de la placa dental]

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Abstract

Context: Dental caries is an infectious disease resulting in destruction of tooth structure by acid-forming bacteria found in dental plaque and intraoral biofilms, which are made up of mixed-species microbial communities, and their uncontrolled outgrowth can lead to oral disease.

Aims: To analyze new biological materials (papain, pectin, three plant extracts and their combinations), for prevention, control, and treatment of oral bacteria and biofilm *in vitro* and *in vivo*.

Methods: Papain, citric pectin, extracts of *Carya illinoinensis, Quercus rubra*, and *Smilax glyciphylla* were applied. *In vitro* test was performed by means of the spectrophotometric assay and CFU evaluation after treatments application. *In vivo* tests were performed to evaluate the number of microorganisms presented in dental biofilm: before and 1.5 h after brushing with different treatments; after 10 days of brushing with various treatments in 10 groups of patients, signing an informed consent approved by the Institutional Ethics Committee of the Autonomous University of Coahuila.

Results: In vitro, the plant extracts inhibited the growth of *Streptococcus* sp. as well as a mixture of microorganisms that form dental biofilms. Papain activity was inhibited by plant extracts. *In vivo*, brushing of teeth with selected plant extracts reduced the number of bacteria in the dental plaques.

Conclusions: The extracts of *Quercus rubra, Carya illinoinensis* and *Smilax glyciphylla* and papain (with or without pectin) had an inhibitory effect on the dental biofilm formation. *In vitro* test demonstrated the bacteriostatic effect of plant extracts or their mixture.

Keywords: Aqueous extract; *Carya illinoinensis*; oral microorganisms control; papain; pectin; *Quercus rubra*; *Smilax glyciphylla*.

Resumen

Contexto: La caries dental es una enfermedad infecciosa; destruye la estructura dentaria por bacterias formadoras de ácido existentes en la placa dental y la biopelícula intraoral, compuestos por comunidades de distintas especies microbianas, la reproducción descontrolada de dichos microorganismos puede causar diversas enfermedades en la cavidad oral.

Objetivos: Analizar nuevos materiales biológicos (papaína, pectina, tres extractos de plantas y sus combinaciones), para la prevención, control y tratamiento de las bacterias orales y biopelícula bacteriana *in vitro* e *in vivo*.

Métodos: Se aplicaron papaína, pectina cítrica, extractos de *Carya illinoinensis*, *Quercus rubra*, y *Smilax glyciphylla*. La prueba *in vitro* se realizó por espectrofotometría y CFU después de la aplicación de tratamientos. *In vivo* se evalúo número de microorganismos en la biopelicula; antes, 1.5 h después del cepillado y después de 10 días de cepillado con los diferentes tratamientos en 10 grupos de pacientes, firmando consentimiento aprobado por el Comité Institucional de ética de la Universidad Autónoma de Coahuila.

Resultados: In vitro, los extractos vegetales inhibieron el crecimiento de *Streptococcus* sp. así como la mezcla de microorganismos que forman la biopelícula bacteriana. La actividad de la papaína fue inhibida por extractos de plantas. *In vivo,* el cepillado de los dientes con extractos de plantas seleccionadas redujo el número de bacterias en la placa dental.

Conclusiones: Los extractos de *Quercus rubra, Carya illinoinensis* y *Smilax glyciphylla* y papaína (con y sin pectina) tuvieron efecto inhibitorio en la formación de la biopelícula dental. Las pruebas *in vitro* demostraron efecto bacteriostático de los extractos de plantas o sus mezclas.

Palabras Clave: *Carya illinoinensis*; control de microorganismos orales*;* extracto acuoso; papaína; pectina; *Quercus rubra*: *Smilax glyciphylla*.

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INTRODUCTION

Dental caries is an infectious disease resulting in destruction of tooth structure by acid-forming bacteria found in dental plaque and intraoral biofilms (Hajishengallis et al., 1992; Berg et al., 2001; Gregorio Jáuregui et al., 2009). Oral biofilms are found on teeth, tongue, cheek tissues and on all mucous membranes of the oral cavity. Oral biofilms are made up of mixed-species microbial communities, and their uncontrolled outgrowth can lead to oral diseases (Stewart and Costerton, 2001; Donlan and Costerton, 2002; Sanclement et al., 2005; Hassan et al., 2011). The bacteria present in the oral cavity may be streptococci, lactobacilli, staphylococci, corynebacteria, and some anaerobes in particular bacteroides. These bacteria colonize the oral mucosa and under appropriate conditions form biofilms on teeth. Biofilm formation by bacteria adhesion is due to physico-chemical and biological processes (Bryers and Ratner, 2006). Biofilm development is due to different processes (Berg et al., 2001) as are: Pre-meditated adsorption of fluid phase organic molecules or substratum pre-conditioning by circumstantial (Johnston et al., 2005), cell desorption from the substratum (De Las Heras Alarcon et al., 2005), bacterial cell transport to the surface (Ista et al., 1999), permanent cell adhesion to the substratum (Kikuchi and Okano, 2002), bacterial metabolism (LaVan et al., 2003), among others.

Some the key processes controlling biofilm formation provide targets for application of novel preventive or remedial technologies. It is known that colonizing bacteria adhere to the protein films of the enamel through specific and nonspecific mechanisms of adhesion. Protease treatments degrade enzymatically adhesion proteins to inhibit biofilm formation (Sato et al., 1983; Johansen et al., 1997; Berg et al., 2001).

In this study, we evaluated the effect of vegetable protease (papain) on the viability of isolated oral bacteria and bacterial biofilm. To immobilize this protease, as well as to modify the teeth surface, pectin was applied. We also evaluated the effect of plants extract on prevention of bacterial biofilms formation. Mexican plants extracts: from barks of walnut tree (*Carya illinoinensis*) and northern red oak (*Quercus rubra*), as well as from leaves and stems of sarsaparilla (*Smilax glyciphylla)* were mentioned in naturist medicine for their ability to control buccal alterations and thus were selected for the research (Monroy and Castillo, 2007). Hence, we studied new biological materials (papain, pectin, three plant extracts and their combinations), as possible candidates for prevention, control, and treatment of oral bacteria and bacterial biofilm in *in vitro* and *in vivo* tests.

MATERIAL AND METHODS

Plant material and reagents

Three plants of the Coahuila State region were used: bark of *Carya illinoinensis* (K. Koch 1869) of the family *Juglandaceae* native of North and Central México, *Quercus rubra* of the family *Lobatae* and leaves and stem of *Smilax glyciphylla* of the family *Smilacaceae* (Hurrel et al., 2011). The plant material was authenticated by the taxonomist of the herbarium of Agrarian University Antonio Narro. Voucher specimens of *C. illinoinensis* (No. 25352), *Q. rubra* (No. 07425) and *S. glyciphylla* (No. 48136) were preserved in the herbarium of agrarian University Antonio Narro, México.

The barks, leaves or stems of plants were dried in shadow at room temperature (25-30°C), ground into powdered form and stored in airtight containers.

In all cases the water infusion (tea) was prepared from dried bark of *C. illinoinensis* (15.99 g/L), dried bark of *Q. rubra* (18.52 g/L), dried leaves and dried stems of *S. glyciphylla* (11.84 g/L) in one liter of water at boiling temperature during 30 min and filtered through filter paper. All the extracts were sterilized by filtering through a 0.22 µm membrane filter (Millipore, México). The quantity of nonvolatile solid compounds was estimated gravimetrically after water sublimation from 100 mL of extract by lyophilization in a freeze dry with capacity of -105°C/4.5 L (FreeZone Labconco, Kansas City).

Stock solutions of pectin and papain were prepared using distilled sterilized water. Both reagents were provided by PROQLIMS (Saltillo, Mexico).

In vitro assays

Two *in vitro* tests were carried out: 1) taking into account the wide variety of bacteria that are forming in the dental biofilm sample it was used with all microorganisms; 2) then use only strain *Streptococcus* sp. previously isolated from the dental biofilm. In both trials, the absorbance increase at 560 nm was measured.

Determination of the minimal inhibitory concentration against dental biofilm microorganisms

The inoculum was prepared (Hertiani et al., 2011; Liu et al., 2013) by a collection of microorganisms from a dental biofilm of three patients (to which were given to it to sign an Informed Consent approved by the Ethics Committee of the Autonomous University of Coahuila) and their proliferation in 20 mL of nutrient broth (Bioxon, México). Microorganisms were grown at 37°C, 5% CO_2 for 12 hours in an anaerobic jar.

Minimal inhibitory concentration (MIC) of plant infusions against oral microorganisms (*Streptococcus* sp.) was determined by broth dilution method as described by Basri et al. (2012). The supplied media were prepared using different dilutions of obtained extracts. Inoculums optical density was adjusted to McFarland standard 0.5. The tubes were then incubated at 37°C, 5% CO_2 for 24 h in an anaerobic jar. The tests were performed in triplicate for each extract. The lowest concentration of the extracts that did not show any increase in absorbance compared to the control tubes after 24 h incubation was reported as MIC (Das et al., 2011).

Kinetic study with Streptococcus *sp.*

Streptococcus sp. isolated from dental plaque microorganisms, using blood-agar base supplemented with 5% of rabbit blood as a differential medium, was used as inoculum. The genus was confirmed by standard biochemical tests (Hajishengallis et al., 1992). The inoculum was prepared from culture propagated in nutrient broth for 10 h as McFarland standard 0.5 (Scott, 2011). To perform the kinetic study, the inoculum (0.2 mL) was added to each tube (from HACH spectrophotometer kit, México) containing 8 mL of the culture medium. To prepare the culture, the two-fold concentrated broth was diluted with an equal volume (8 mL) of plant extracts, pectin or, papain solution, and pectin-papain mixture (1:1). Culture media were sterilized though filtration (0.22 μm Millipore). Thus, final dilutions of extracts were at a concentration of 20%, pectin and papain concentrations were 1 and 2% (w/v), respectively. The commercial toothpaste was used at 0.5% (w/v) as a final concentration in the nutrient medium, as a positive control.

Immediately after adding inoculum to each tube, the absorbance at 560 nm corresponding to zero h of incubation was measured in a spectrophotometer (HACH, México) and then the tubes were placed under microaerophilic condition at 37°C. The absorbance was measured at defined time points during 14 h of incubation at 37°C. The difference in absorbance between time zero and other time points was plotted against time of incubation.

Effect of plant extracts on papain activity was evaluated in triplicate by Kunitz´s technique (Kunitz, 1947; Gregorio Jáuregui et al., 2009). The enzyme was pre-incubated for 5 min with different dilutions (10, 20, 30, 40, 50 y 60%) of the extracts to compare the activity with control carried out using water instead of the extracts (Kunitz, 1947; Gregorio Jáuregui et al., 2009).

In vivo assays

These assays were authorized by the Institutional Ethics Committee of the Faculty of Medicine of the Autonomous University of Coahuila.

In vivo tests were performed to evaluate the number of microorganisms presented in dental biofilm: 1) before and 1.5 h after brushing with different treatments; 2) after 10 days of brushing with various treatments (the last brushing was approximately 10 h before the evaluation). Both *in vivo* tests were carried out in 10 groups of patients (10 patients in each group), who signed a

form approved by the Institutional Ethics Committee of the Autonomous University of Coahuila consent; where the procedure, the purpose of the test and the possible results would favorable the study explained, complying with the ethical principles adopted in the Declaration of Helsinki.

The tests were conducted in a double-blind, randomized, placebo-controlled design (water). The negative and positive control treatments were: water as placebo, water sweetened with artificial sweetener (1.0 g) and toothpaste 1% (w/v) (composition: humectant and water 75%, abrasives 20%, foam and flavorings 2%, buffers 2%, coloring agents, binders opacifiers 1.5% and fluoride 0.10-0.15%), respectively. The following treatments were tested:

1) Water (10 mL),
2) Pectin gel 6% (w/v),
3) Papain solution 2% (w/v);
4) Pectin-papain mixture 6% and 2% (w/v),
5) *Quercus rubra* bark 10% (w/v),
6) *Carya illinoinensis* bark 10% (w/v),
7) *Smilax glyciphylla* leaves and stems 10% (w/v)
8) Mixture of each extract at a ratio of 1:1:1 from a concentration of 10%,
9) Mixture of each extract with pectin-papain used in group 4 (1:1),
10) Toothpaste 1.1% (w/v).

The first test was performed to evaluate the number of microorganisms before and 1.5 h after brushing with treatment randomly selected by the patient. Treatment was carried out according to the common procedure: brushing for 2 min using the treatment, following two subsequent rinsing with water. To quantify the number of microorganisms before and after treatment, CFU/mL were evaluated, using blood-agar as culture medium (with 5% of rabbit blood). In each case, calibrated inoculating loop was used to suspend the removed dental plaque in 10 mL of sterile peptone water (1.5%). The suspension was vortexed well for 3 min, and then 10 and 1000 fold dilutions were prepared. An aliquot of 1 mL of each dilution was added to blood-agar plates. The Petri dishes were incubated at 37°C under

microaerophilic condition (approximately 7% of oxygen, 10% of carbon dioxide, and room air); after 24 h of incubation the number of colonies was counted in each dish. All tests were performed in triplicate.

In the second test the same procedures of treatments were performed by patients three times daily for 10 days (brushing the schedule was carried out as follows; waking brushing is not held and the sample plate is taken, then brushing teeth and 1.5 h had a sample plate was taken, and finally held a brushed three times a day for 10 days mainly after meals with selected treatment; at the end of 10 days of brushing returned to take a sample of the plate). The last treatment was applied approximately 10 h before microbiological assay. The CFU/mL quantification was carried out as described above. Visual inspection of alterations of oral tissues, with unusual presence of inflammations and some pain (or negative sensations of patient), also was carried out

Statistical analysis

Analysis of variance (ANOVA) was conducted among sample means of each treatment, test was used to evaluate the differences (α = 0.01). p < 0.01 according to Tukey's test was considered statistically significant. SAS software version 9 (SAS Institute Inc. USA) was used for analysis of all data.

RESULTS

Obtaining extracts

The extracts obtained in five extractions were characterized by a reproducible content of soluble, nonvolatile compounds obtained in a relatively high percentage of total yields (Table 1).

In vitro assays

The extracts had an acceptable taste according to persons, which voluntarily tested them. MICs of plant extracts against a mixture of oral microorganisms are specified in Table 2. The detected MIC values indicated that the extract had antibacterial activity.

Streptococcus sp. growth kinetics in the presence and absence of applied treatments are presented in Figs. 1 and 2. Almost all applied treatments, excepted papain and toothpaste, led to a decrease of *Streptococcus* sp. growth. Fig. 3 shows linearization of exponential growth phase in semi-logarithmic coordinates in case of six systems described by this behavior. The slopes of the obtained lines are considered as the specific growth rate of *Streptococcus* sp.

The kinetics in the presence of papain was characterized by an increase of maximum absorbance level without significant changes in specific growth rate compared to control (Figs. 1 and 3). Growth kinetics in presence of toothpaste was not characterized by a detectable exponential phase; rapid absorbance increase to level slightly higher than detected in control assay was observed, followed by its slow decline (Fig. 1B).

In contrast, the addition of pectin, as well as three extracts mixture completely inhibited bacterial growth, what is observed with a decrease in the absorbance values (Fig. 1B and Fig. 2). In the presence of *Q. rubra* extract (Fig. 1A) and the pectin-papain solution mixed with three plant extracts (Fig. 2) slightly increased absorbance due to strong inhibition of bacterial growth. Such as, in case of kinetic detected in the presence of

extract from *Q. rubra*, its linearization in semi-logarithmic coordinate was not possible. The addition of pectin-papain solution mixed with three plant extracts to culture medium led to 2.5 times decrease of specific growth rate and maximum absorbance level in comparison with control (Figs. 2 and 3).

Partial inhibition of *Streptococcus* sp. growth was observed in the presence of *C. illinoinensis* and *S. glyciphylla* extracts, as well as a pectin-papain mixture: the absorbance levels were less than in control assay. The specific growth rate decreased by 1.6 times in comparison with control in the case of pectin-papain mixture and *C. illinoinensis* extract, and was not changed significantly (in all three repetitions) in the presence of *S. glyciphylla* or papain.

To explain the difference between pectin-papain and pectin-papain mixed with three plant extracts effect on bacterial growth, papain enzymatic activity in the presence of plant extracts was evaluated (Fig. 4). The inhibition of enzymatic activity was demonstrated. The inhibitory effect is a function of extract concentration: it increases with extracts concentration increase. *C. illinoinensis* and *S. glyciphylla* extracts demonstrated similar papain inhibition, greater than *Q. rubra* extract.

Table 1. Characteristics of the plant extracts.

Plant extract	Drug weight* (g)	Infusion color	Soluble nonvolatile compounds (total yield, mg/L)	Yield (%)
Carya illinoinensis (bark)	15.99 (16000 ppm)	Orange	2060 ± 119	12.9
Quercus rubra (bark)	18.52 (19000 ppm)	Red	3400 ± 211	18.4
Smilax glyciphylla (leaves and stems)	11.84 (12000 ppm)	Yellow	1700 ± 150	14.4

The data represent mean ± SD of five experiments. The infusions were prepared with the vegetal drugs in 1 L of water for 2 min. *pinch of corresponded powder was taken and weighted, according with the traditional use.

Table 2. Minimum inhibitory concentration values of aqueous plant extracts.

Plant extract	Mean MIC (mg/L)
Carya illinoinensis	257.5 (8 fold dilution of initial extract)
Quercus rubra	340.0 (10 fold dilution of initial extract)
Smilax glyciphylla	212.5 (8 fold dilution of initial extract)

Minimum inhibitory concentration (MIC) values are the results of microorganism inoculum of three patients with aqueous extracts.
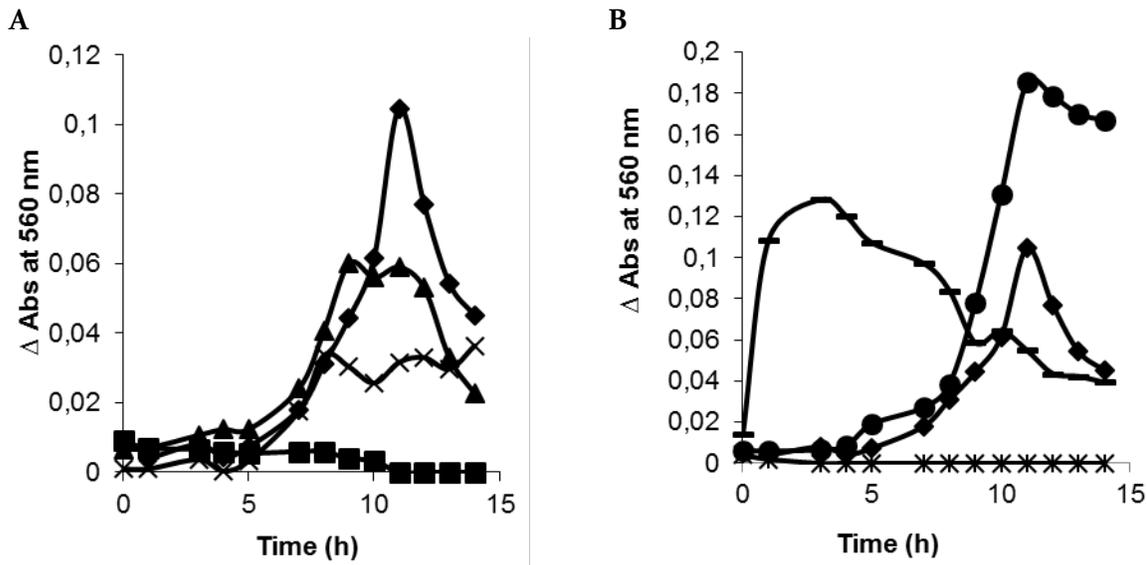
A

B

Figure 1. Kinetics of *Streptococcus* sp. growth in presence and absence (control -♦-) of extracts from: **A:** - *Q. rubra* 10% (-■-), *C. illinoinensis* 10% (-▲-), *S. glyciphylla* 10% (-x-); **B:** - pectin at 1% (-ж-), papain at 2% (-●-) and toothpaste at 0.5% (- - -). The results are expressed as mean of n = 3.
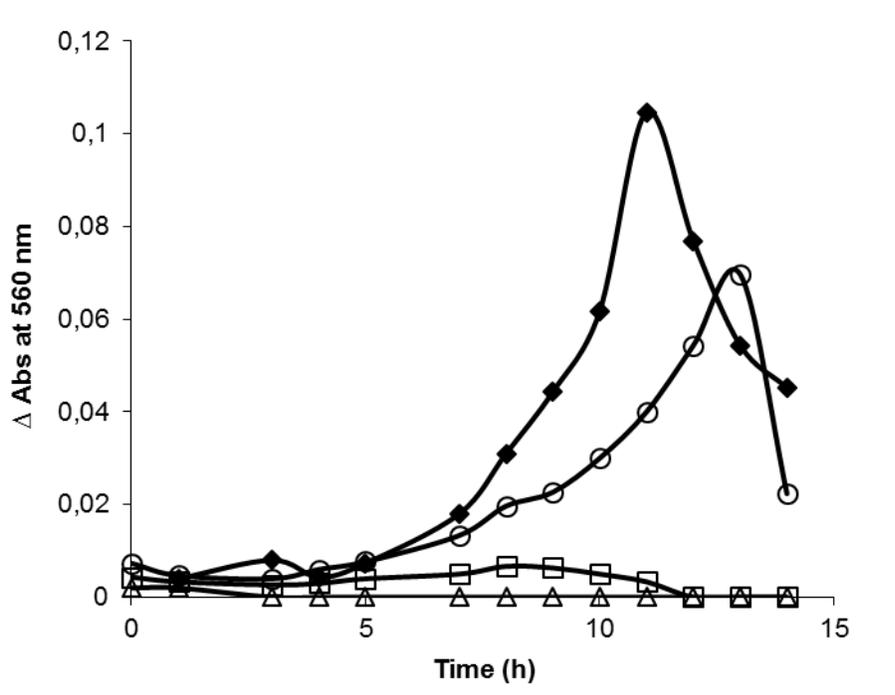

Figure 2. Kinetics of *Streptococcus* sp. Growth, performed in triplicate in the presence and absence (control-♦-) of pectin-papain (-○-); extracts mixture (-Δ-) and the same with pectin-papain (-□-). The results are expressed as mean of n=3.
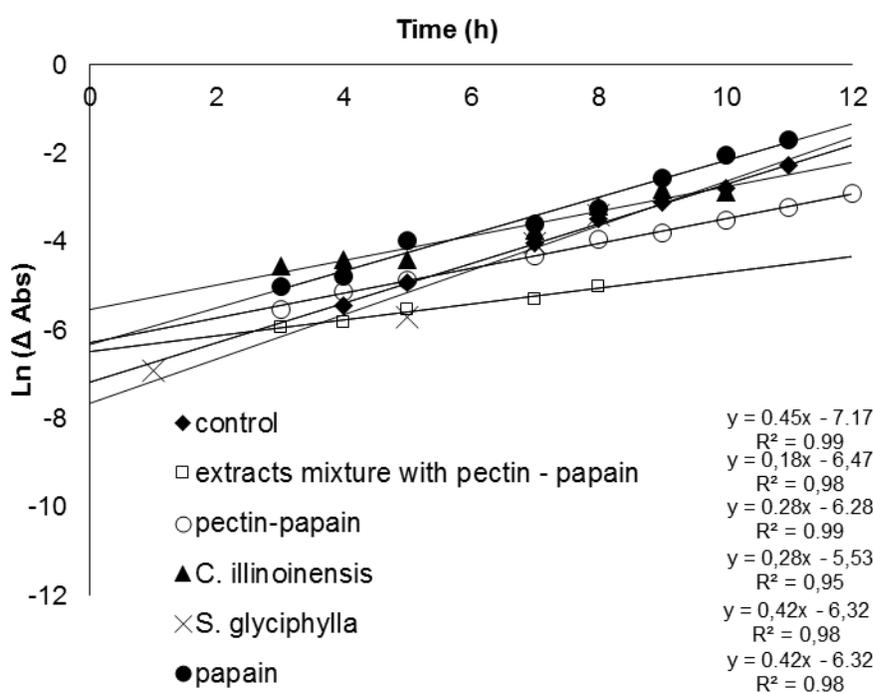
Figure 3. Linearization of the exponential phase of *Streptococcus* sp. growth kinetics (from Figs. 1-2) on semi-logarithmic coordinates. [Estimated specific growth rate values were: 0.45 (Control), 0.18 (extracts mixture with pectin-papain), 0.28 (pectin-papain), 0.28 (*C. illinoinensis*), 0.42 (*S. glyciphylla*), and 0.42 h⁻¹ (papain)]. Results are expressed as mean of n=3.
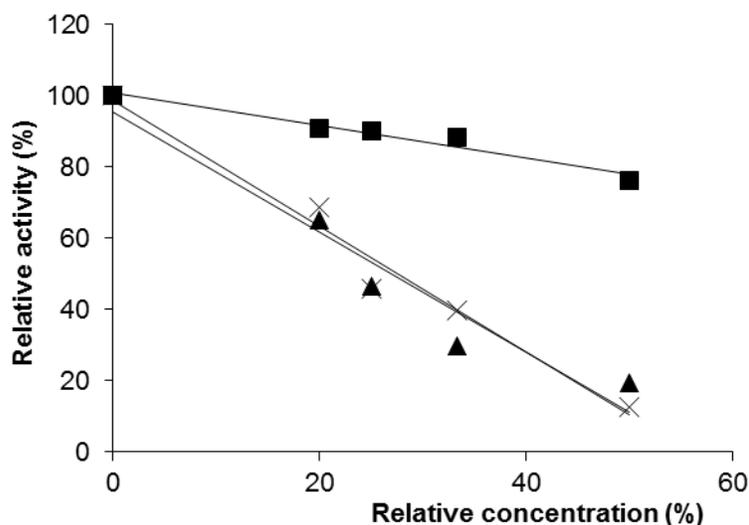

Figure. 4. Effect of different relative concentrations of extracts from: *Q. rubra* (-■-), *C. illinoinensis* (-▲-), and *S. glyciphylla* (-x-) on papain activity. (Activity without extracts was considered 100% of relative activity. Extracts without dilution were taken in account as 100% of relative concentration). Results are expressed as mean of n=3.

In vivo assays

Results of *in vivo* testing are presented in Table 3, wide standard deviations were detected due to the great variety between different patients. Therefore, the descriptive statistic was not applicable in this case, and ANOVA test was performed.

At 1.5 h after application of treatments and brushing (initial state of biofilm formation), the microorganisms quantity was estimated in blood-agar (Table 3). A number of microorganisms was less than detected before treatments application. ANOVA test (Table 3) demonstrated that all applied treatments had a significant difference ($p < 0.01$) in comparison with negative control (brushing with water sweetened with artificial sweetener). However, only in case of *S. glyciphylla* extract the detected values were statistically different ($p < 0.01$) from other treatments (Table 3). The significant difference was not detected among other treatments and conventional treatment with toothpaste ($p > 0.01$).

Later, the patients were asked apply the corresponding treatment for 10 days, three times a day in the brushing schedule. At the end of the established term, the evaluation of the microbial load was performed as described above (Table 3). The standard deviations were very ample. Applying the ANOVA test, it was demonstrated that the major values of microbial load were detected in the case of brushing with water and gel of pectin.

The values of CFU/mL before and after 10 days treatments application were not significantly different (Table 3). These data were similar to detected on the first day of the assay before treatments application (Table 3). With other designed treatments average of microbial load were smaller than the one detected in the presence of toothpaste, but there was no significant difference. Fewer microorganisms able to grow in blood agar were detected in patients using pectin-papain and mixture of three extracts (Table 3). Thus, *in vivo* test demonstrated that the designed treatments were better than brushing with only water and similar or in some case better than brushing with toothpaste.

Table 3. The amount of microorganisms in dental biofilm before 1.5 h after brushing and after 10 days with different treatments application.

Treatment	CFU/ mL ± SD in blood–agar	CFU/ mL ± SD in blood–agar
Initial state [last brushing with toothpaste at previous night (approximately 10 h)]	886 ± 236[a]	
	Evaluation at 1.5 h after brushing with treatment	**Evaluation at 10 days after with treatment**
Water (10 mL)	112 ± 65[a]	1085 ± 409[a]
Pectin gel (6%)	13 ± 8[b]	1036 ± 380[a]
Papain (2%)	22 ± 11[b]	598 ± 324[b]
Q. rubra extract (10%)	14 ± 6[b]	798 ± 439[b]
C. illinoinensis extract (10%)	14 ± 8[b]	526 ± 256[b]
S. glyciphylla extract (10%)	4 ± 3[c]	577 ± 148[b]
Pectin-papain (6%-2%)	11 ± 5[b]	478 ± 237[b]
Mixture of three extracts (1:1:1)	13 ± 8[b]	408 ± 225[c]
Pectin-papain mixed with three extracts (1:1)	27 ± 12[b]	676 ± 235[b]
Toothpaste (1.1%)	28 ± 11[b]	745 ± 356[b]

Data from ten independent determinations are expressed as mean ± SD. Treatments not sharing the same letters are significantly different by the Tukey-test ($p < 0.01$) in each group.

DISCUSSION

In recent years, there has been increasing interest worldwide in the use of alternative/herbal medicine for the prevention and treatment of various illnesses (Prashant et al., 2011). Plant products and extracts of various plant parts have been used extensively as natural antimicrobials and antioxidants (Ncub et al., 2008; Das et al., 2011), frequently containing the heterogeneous chemically active component (Akpata and Akinrimisi, 1977). In the analysis stage plants sample preparation is important, since it must extract the desired chemical components of materials for characterization (Monroy and Castillo, 2007; Das et al., 2010). Quantity of soluble, nonvolatile compounds extracted in five assays using different samples of the herbal material varied less at 8% (Table 1), that may be considered as criteria of reproducibility of the extraction process. The percentage of soluble, nonvolatile solids (Table 1) was higher than reported for extracts from *Myristica fragrans* and *Quercus infectoria* (Basri et al., 2012). However, extracts obtained from assayed plants (Table 2) were less potent than these two plant extracts and standard antibiotics, such as tetracycline or metronidazole, to which the different pathogenic bacteria were highly susceptible at 5 µg/mL (Basri et al., 2012).

Growth inhibition of an oral microorganism's mixture (found in dental plaque and may be responsible for cariogenic or periodontal dama-ges) as well as *Streptococcus* strain used in the present study (Table 1 and Figs. 1-3, respectively) was observed. Perhaps it is related to the effect of antibacterial metabolites present in the obtained extracts. It is well-known that plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have anti-microbial properties (Cowan, 1999). *Q. rubra, C. illinoinensis* and *S. glyciphylla* leaves and stems extracts used at the same concentration showed a bacteriostatic effect against *Streptoco-ccus* sp. (Fig. 1A). In this case, the microorganism growth was inhibited only after some hours of proli-feration, which comprises the existence of a

maximum of absorbance in the kinetic curves. Inhibition was greater in the case of *Q. rubra* extract (Fig. 1A). It was demonstrated that the inhibitory effect was related to decrease of specific growth rate, as well as to decrease of maximum absorbance level in case of *C. illinoinensis* extract and the mixture of three extracts with pectin-papain (Fig. 3). However, in *S. glyciphylla* extract the decrease of specific growth rate was not significant ($p > 0.01$), and the inhibitory effect was observed as a decrease of maximum absorbance change. The mixture of three extracts totally inhibited the growth of this microorganism, although at concentrations lower that each one separately (Figure 2). The potentiation of medicinal effect in the presence of plant extract mixtures was reported in various studies (Cowan, 1999).

The different effect of pectin, papain and pectin-papain was observed. The *Streptococcus* sp. growth was totally inhibited with pectin, partially with pectin-papain, and was increased in the presence of papain. The pectin effect may be explained by a rise in medium viscosity or decrease of water activity (Gregorio Jáuregui et al., 2009). Papain is a well-known proteolytic enzyme from papaya (*Carica papaya*). *C. papaya* yields a milky sap, often called latex, widely used in Latin American and African countries as an oral hygiene aid (instead of a toothbrush). Latex is a complex mixture of chemicals, chief among them is papain; an alkaloid, carpaine, is also present (Burdick, 1971). Terpenoids are also present and may contribute to its antimicrobial properties. Osato et al. (1993) found that the latex has a bacterios-tatic effect on some *Enterobacteriaceae* (*Entero-bacter cloacae, Escherichia coli, Salmonella typhi*), and Coco bacilli (*Staphylococcus aureus, Bacillus subtilis*) (El-Kholy, 2008). In the present study, papain was tested *in vitro* against *Streptococcus* sp. (Fig. 1), and the growth inhibition was not observed. On the contrary, in nutrient liquid media papain activity led to increasing in *Streptococcus* sp. growth. The specific growth rate is not changed (Fig. 3), while the absorbance level increases (Fig. 2). Probably papain hydro-lyzes the proteins presented in the nutrient broth, which may facilitate the microbial growth

(Norton and Addy, 1989). Papain inhibition with plant extracts mixture (Fig. 4) decreased its ability to promote *Streptococcus* sp. growth in liquid nutrient broth. Thus, papain did not demonstrate antibacterial effect against *Streptococcus* sp. in a liquid medium. However, it is useful in assays with biofilm formation due to protease hydrolysis of adhesion proteins (Van Palestein Helderman et al., 1983; De Las Heras Alarcon et al., 2005). Ledder et al. (2009) reported that while papain inhibited co-aggregation in binary assays, this protease, as well as amylase and lipase treatments did not significantly alter consortium population dynamics. Moreover, *in vitro* testing of commercially available toothpaste also did not demonstrate the bactericidal effect, only slightly bacteriostatic effect. In this test, the toothpaste concentration was decreased to 0.5% (w/v) to avoid its influence on absorbance detection. It might be the cause of its non-efficient performance.

It is well-known that bacteriostatic and bactericidal effect of natural treatments is specific against particular bacterial strains (Basri et al., 2012): extracts and their components inhibit some bacteria, as *Streptococcus* sp. strain. Of course, plants have been used for centuries to treat infections and other illnesses in humans in aboriginal groups but controlled clinical studies are scarce (Cowan, 1999). In West Africa, it conducted a cross-sectional epidemiological study involving the effectiveness of chewing sticks in front of toothbrushes for oral hygiene (Cowan, 1999). The authors found a reduced effectiveness in chewing-stick users compared to toothbrush users and concluded that the antimicrobial chemicals known to be present in the sticks added no oral health benefit (Norton and Addy, 1989). Also, regarding oral health, mouthrinses containing various antimicrobials have been evaluated in humans (Walker, 1988). Mouthrinses containing phytochemicals were not found to be as effective in decreasing plaque or clinical gingivitis as were Listerine or chlorhexidine. Due to these previous findings, in the present study toothbrushes were used to all treatments application *in vivo* testing.

The blood-agar base supplied with 5% of rabbit blood was used because inhibition effect was observed *in vitro* test with potentially pathogenic microorganism isolated from buccal biofilm applying this medium. Blood agar (BA) is an enriched medium that provides an extra rich nutrient environment for microbes. Therefore, BA is not a selective growth medium since it supports the growth of a broad range of organisms. BA is a differential growth medium. A growth medium is considered differential if, when specific microbes are present, the medium or bacterial colonies themselves exhibit a color change that provides information about their identity. Certain bacteria produce exotoxins called hemolysins, which act on the red blood cells to lyse, or break them down. So, the hemolytic microaerophilic bacteria (as *Streptococcus*) growth was expected when using BA medium under applied conditions.

First, the sample of biofilm microorganisms was taken with gauge handles 1.5 hours after treatments application. This period was selected because the dental plaque begins its formation 45 minutes after the brushing (Daly, 2009). This was the intention to evaluate the effect of treatments at the early stage of the biofilm formation. It was observed that after brushing the amount of microorganisms present in the dental surface was diminished considerably (Table 4). This is the consequence of the toothbrush mechanical intervention that by many years has been considered a useful method for teeth care (Smits and Arends, 1985). A wide range of standard deviation demonstrated that patients have a different susceptibility to applied treatments and ability to buccal biofilm formation. Even so, the statistically significant difference was observed between values corresponding to the control and other applied treatments, including pectin gel. The statistically significant difference was not detected among most other treatments and conventional treatment with toothpaste, except *S. glyciphylla* extract, with which the lowest bacterial count was detected. Pectin gel forms a film (Gregorio Jáuregui et al., 2009), which probably retards the addition microorganisms. Papain probably acts by hydrolysis of adhesion proteins (Dawkins et al., 2003), while plants extracts perform their effect using their antibacterial metabolites (Cowan, 1999).

After long-time treatments application, the similar tendency was observed, except to pectin gel with which the results similar to control (brushing with sweet water) were obtained. Probably microorganisms could adapt to the presence of pectin film. The greatest statistically significant microbial inhibitions were detected in the case of pectin-papain and three extracts mixture. Potentiation of the inhibitory effect was also demonstrated while pectin film led to maintain papain on the dental surface. Thus, *in vivo* testing shows that treatments containing extracts or papain were effective in decreasing oral biofilm.

CONCLUSIONS

According to results of *in vivo* testing, extracts of the selected plants (*Quercus rubra*, *Carya illinoinensis* and *Smilax glyciphylla*) and papain (with or without pectin) had an inhibitory effect on the dental biofilm formation. The use of selected treatments can replace dental grazes, at least for 10 days since it allows controlling the formation of dental biofilm. *In vitro* test demonstrated the bacteriostatic effect of plant extracts or their mixture. However, *in vitro* assay performed with nutrient broth was not appropriate to observe the inhibitory capacity of proteases or toothpaste.

Thus, brushing of teeth with selected plant extracts reduced the number of bacteria in the dental plaques, suggesting that the inclusion of certain plant extracts into dental hygiene products could reduce bacterial biofilm formation thereby control dental caries.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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